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Cofactor Engineering: a Novel Approach to Metabolic Engineering in *Lactococcus lactis* by Controlled Expression of NADH Oxidase

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NADH oxidase-overproducing *Lactococcus lactis* strains were constructed by cloning the *Streptococcus mutans* *nox-2* gene, which encodes the H₂O-forming NADH oxidase, on the plasmid vector pNZ8020 under the control of the *L. lactis* *nisA* promoter. This engineered system allowed a nisin-controlled 150-fold overproduction of NADH oxidase at pH 7.0, resulting in decreased NADH/NAD⁺ ratios under aerobic conditions. Deliberate variations on NADH oxidase activity provoked a shift from homolactic to mixed-acid fermentation during aerobic glucose catabolism. The magnitude of this shift was directly dependent on the level of NADH oxidase overproduced. At an initial growth pH of 6.0, smaller amounts of nisin were required to optimize NADH oxidase overproduction, but maximum NADH oxidase activity was twofold lower than that found at pH 7.0. Nonetheless at the highest induction levels, levels of pyruvate flux redistribution were almost identical at both initial pH values. Pyruvate was mostly converted to acetoin or diacetyl via α -acetolactate synthase instead of lactate and was not converted to acetate due to flux limitation through pyruvate dehydrogenase. The activity of the overproduced NADH oxidase could be increased with exogenously added flavin adenine dinucleotide. Under these conditions, lactate production was completely absent. Lactate dehydrogenase remained active under all conditions, indicating that the observed metabolic effects were only due to removal of the reduced cofactor. These results indicate that the observed shift from homolactic to mixed-acid fermentation under aerobic conditions is mainly modulated by the level of NADH oxidation resulting in low NADH/NAD⁺ ratios in the cells.

Lactococcus lactis strains are used worldwide in the manufacture of dairy products and have the potential to produce a variety of end metabolites during sugar fermentation (8). Some of these compounds, such as diacetyl, acetaldehyde, or extracellular exopolysaccharides, have a great economic importance because of their contribution to flavor or texture development. However, product formation from sugars in *L. lactis* is generally homolactic. Metabolic shifts leading to end products other than lactate, the so-called mixed-acid fermentation, have been observed under certain fermentation conditions, such as utilization of galactose as the sole carbon and energy source (28), carbohydrate limitation (27), or aerobic conditions (1, 5). It has recently been pointed out that diminished rates of sugar metabolism led to shifts from homolactic to mixed-acid fermentation, while rapid flux through the central pathways resulted in homolactic fermentation (4, 9, 11). In apparent contrast, shifts towards mixed-acid fermentation have also been observed at high imposed glycolytic fluxes during metabolism under aerobic conditions (19). The direct oxidation of the NADH necessary for pyruvate reduction resulted in a diminished flux towards lactate via LDH. The ratio of NAD⁺ to NADH, used as an indicator of the redox state of the cells, was directly affected, at the expense of oxygen, by the NADH oxidase activity, which mainly determined the observed shift. This enzyme activity was found to be induced in *L. lactis* under conditions that showed the most pronounced shifts, such as a high dilution rate and low pH values.

Recently it has been shown that sugar metabolism in *L. lactis* also may be manipulated by using metabolic engineering at the

level of the central intermediate pyruvate (8, 22). Different levels of flux redistribution have been obtained, depending on the engineered branchpoint of the network. However a combination of several strategies, i.e., overproduction of α -acetolactate synthase (ALS) in an *L. lactis* strain deficient in LDH, have rendered larger pyruvate flux redistribution than single modifications of one enzyme's activity (22). This appears to be a common feature in modifying metabolic networks: large effects on flux redistribution are obtained when more than one enzyme is engineered (21).

In this paper, we describe a new strategy to modify metabolic flux in *L. lactis*, by using metabolic engineering on the level of NADH oxidation. This modification was performed by controlled overproduction of NADH oxidase activity. To the best of our knowledge, this is the first report of manipulation of the level of a key cofactor which is shared by several enzymes involved in the metabolism. We have constructed NADH oxidase-overproducing *L. lactis* strains based on a recently developed nisin-inducible expression (NICE) system (7, 18). Deliberate variations of NADH oxidation levels could be obtained in these strains by fine tuning of NADH oxidase overexpression in such a way that the shift from homolactic to mixed-acid fermentation could be controlled by the addition of subinhibitory amounts of nisin. The results presented here extend our insight in the key role of redox balance on pyruvate metabolism and also describe the application of cofactor engineering for the overproduction of diacetyl, an industrially relevant metabolite.

MATERIALS AND METHODS

Media and cultivation conditions. Unless otherwise indicated, *L. lactis* strains were routinely grown at 30°C in M17 broth (26) (Difco Laboratories, Detroit, Mich.) containing 0.25% (w/vol) glucose (GM17). When needed, HCl was used to set media at an initial pH of 6.0. Fermentations under aerobic conditions were

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performed in duplicate in 500-ml flasks containing fresh GM17 medium (20 ml) with shaking in a G76 water bath (New Brunswick Scientific, Edison, N.J.) at 250 rpm. The medium was supplemented with chloramphenicol ($5 \mu\text{g ml}^{-1}$) and, if appropriate, flavin adenine dinucleotide (FAD [$2 \mu\text{g ml}^{-1}$]). For controlled expression of NADH oxidase, nisin (0.1 to 1.6 U ml^{-1}), purified from a crude batch (NS764; Sigma, Zwijndrecht, The Netherlands) containing 2.5% nisin A, was freshly supplemented to the medium at the start of fermentation.

Plasmid constructions and strains used. The *Sorpirococcus mutans* *nox-2* gene, encoding a water-forming NADH oxidase, was previously identified and cloned on a plasmid designated pSSU61 (20). The *nox-2* gene was amplified by PCR with *Taq* polymerase (Gibco-BRL, Breda, The Netherlands) with pSSU61 as a template and the primers P6 (5'-ATAGGATCCCGTTTCAACCTCATGCTA-3') and P8 (5'-ATAGAGCTCTTTTCACTGTTTCATTTCATAA-3'), which are based on the sequence published previously (20) and are designed to introduce *Bam*HI and *Sst*I restriction sites (underlined in the primer sequences) upstream and downstream of the *nox-2* coding region, respectively. A PCR product with the expected size (1,467 bp according to the sequence previously published [20]) was obtained and cloned as a *Bam*HI-*Sst*I fragment into the similarly digested, high-copy-number (≈ 50) shuttle vector pNZ8020 under control of the *nisA* promoter, with *Escherichia coli* MC1061 as a host (6, 7, 16, 22). Subsequently, the resulting plasmid, designated pNZ2600, was introduced in *L. lactis* NZ9800 (17), which allowed nisin-controlled expression of the *nox-2* gene. Previously, it has been shown that this NICE system exhibits a linear dose-response relationship between the inducer (nisin) concentration and the level of expression of the gene cloned under transcriptional control of the *nisA* promoter (6, 7, 16).

Analysis of growth and fermentation products. Growth and growth rate were determined by measuring the increase in optical density at 600 nm (OD_{600}). Acetate, formate, lactate, ethanol, butanediol, and residual glucose were measured by high-performance liquid chromatography (HPLC) as described previously (15). The products α -acetylactate, acetoin, and diacetyl were measured colorimetrically as described previously (15).

Enzyme analysis. Cells from duplicate batch cultures were harvested at the end of the stationary growth phase (OD_{600} of 1.3). Cell extracts were prepared from the pellets with a bead beater (Biospec Products, Bartlesville, Okla.) as described previously (19). NADH oxidase activity in cell extracts was assayed spectrophotometrically at 25°C in a total volume of 1 ml containing 50 mM potassium phosphate buffer (pH 7.0), 0.29 mM NADH, and 0.3 mM EDTA. The reaction was initiated by the addition of a suitable amount of extract (0.5 to 5 μl) and monitored by the decrease in A_{340} . A unit of enzyme was defined as the amount which catalyzed the oxidation of 1 μmol of NADH to NAD per min at 25°C. L-LDH activity was assayed for the same extracts by the method of Hillier and Jago (12). Protein concentrations were determined according to the Bradford method (3) with bovine serum albumin as a standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize NADH oxidase overproduction in *L. lactis* NZ9800 cells harboring pNZ2600 upon induction with different nisin concentrations. Electrophoresis was carried out at room temperature at 200 V for 1.5 h with a 3.75% acrylamide stacking gel over a 7.5% acrylamide resolving gel (0.75 mm thick). A vertical electrophoresis apparatus (Mini-Protein II) and all electrophoresis reagents were purchased from Bio-Rad (Veenendaal, The Netherlands), except for prestained molecular mass standards (low range), which were purchased from Gibco-BRL (Breda, The Netherlands). Methods for casting gels, electrophoresis, preparing buffers, and staining proteins with Coomassie blue were performed as described in the manufacturer's recommendations (Bio-Rad).

NADH/NAD ratio. NADH and NAD levels were measured as described previously (23). Cells were cultivated in M17 medium with an initial pH of 7.0, with and without 1.2 U of nisin per ml, aerobically and anaerobically and harvested in the early exponential phase.

RESULTS

Cloning and overexpression of *S. mutans* *nox-2* in *L. lactis* NZ9800. By using the previously described NICE system (16), the PCR-amplified *S. mutans* *nox-2* gene was cloned in *L. lactis* NZ9800 (for detail, see Materials and Methods). Extracts from cells of strain NZ9800 harboring pNZ2600 and grown aerobically at an initial pH of 7.0 were used to determine the specific activity of NADH oxidase after induction with increasing concentrations of nisin and in the absence of the inducer. Addition of 0.2 U of nisin ml^{-1} increased NADH oxidase activity up to $3.23 \text{ mmol mg}^{-1} \text{ min}^{-1}$, which is a 16-fold increase compared with the level in noninduced cells (Table 1). Overproduction of NADH oxidase was directly dependent on the amount of nisin added and reached its maximal value upon induction with 1.2 U of nisin ml^{-1} (Table 1). This maximal activity was 150-fold higher than that found in noninduced cells. Surprisingly, NADH oxidase activity decreased after nisin addition levels

TABLE 1. Glucose fermentation pattern of *L. lactis* NZ9800(pNZ2600) at initial growth pH of 7.0

Amt of nisin added (U/ml)	Final pH	Amt of product produced (mM)			Amt of NADH oxidase overproduced (U/mg)*
		Lactate	Acetate	Acetoin or diacetyl	
0	5.96	21.1	5.4	ND*	0.20
0.2	6.26	17.8	8.1	2.7	3.23
0.4	6.40	12.0	9.1	4.8	5.70
0.8	6.44	8.6	9.4	6.8	12.79
1	6.44	6.8	8.3	7.7	16.58
1.2	6.46	5.4	8.4	8.5	29.95
1.4	6.44	6.9	8.1	7.6	17.68

* Overproduction of the heterologous H_2O -forming NADH oxidase at an initial growth pH of 7.0.

* ND, not detected.

higher than 1.2 U ml^{-1} , which could indicate the presence of a shutoff mechanism at high nisin concentrations. The observed results were highly reproducible, as could be seen by the maximum of 10% difference in enzyme activity between the duplicate samples. Since diacetyl or acetoin production (via acetylactate) is optimal at pH 6.0 (24), similar growth experiments with *L. lactis* NZ9800 harboring pNZ2600 were performed at an initial growth pH of 6.0. Addition of only 0.1 U of nisin ml^{-1} rapidly induced an increase in NADH oxidase activity 40-fold higher than that observed in noninduced cells (Table 2). The highest activity reached at this initial pH was 70-fold higher than that found in noninduced controls but twofold lower than the highest value found at pH 7.0. The amount of nisin required to optimize overproduction at an initial pH of 6.0 was 0.4 U of nisin ml^{-1} , which is threefold lower than that required to optimize overproduction at neutral pH. NADH oxidase activity declined when nisin was used at levels higher than 0.4 U ml^{-1} , indicative of the shutoff mechanism described above.

NADH oxidase overproduction in extracts from *L. lactis* NZ9800 cells harboring pNZ2600 induced with different concentrations of nisin was monitored by SDS-PAGE. As expected, the 50-kDa *S. mutans* oxidase was overproduced in *L. lactis* NZ9800 upon induction with nisin at concentrations between 0.1 and 1.4 U ml^{-1} (Fig. 1). The relative amount of oxidase seen on the gels correlated well with the oxidase activity measured. Also, the drop in activity was accompanied by a decrease in protein level as visualized by SDS-PAGE. This result strongly suggests that the observed decrease in activity is due to a progressive switching off of the *nisA* promoter-based system, rather than an inactivation of the overproduced oxidase.

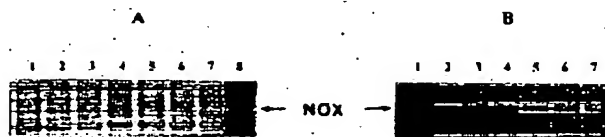


FIG. 1. SDS-PAGE of crude extracts from *L. lactis* NZ9800 harboring pNZ2600 and overproducing the heterologous H_2O -forming NADH oxidase (NOX) at an initial growth pH of 6.0 (A) or 7.0 (B). (A) Lane 1, uninduced cells; lanes 2 to 7, induction with 0.1, 0.2, 0.4, 0.8, 1.2, and 1.4 U of nisin ml^{-1} , respectively. (B) Lane 1, uninduced cells; lanes 2 to 7, induction with 0.2, 0.4, 0.8, 1.2, and 1.4 U of nisin ml^{-1} , respectively. A total of 7.5 μg of protein was applied per well.

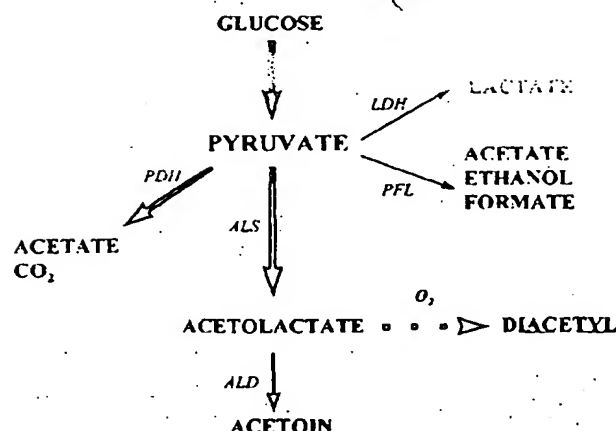


FIG. 2. Schematic pathway of pyruvate metabolism in *L. lactis* (A) and modification of the same pathway after optimization of activity of the overproduced NADH oxidase with FAD (B). ALD, acetolactate decarboxylase.

Metabolic shifts induced by nisin-controlled NADH oxidase overproduction in *L. lactis* NZ9800. Since NADH is the key cofactor in the carbon metabolism of *L. lactis*, metabolic engineering on the level of NADH oxidation should have a marked effect on end product formation. Figure 2 shows the different possibilities of pyruvate conversion that can be found in *L. lactis*. At the initial pH of 7.0, *L. lactis* NZ9800 harboring pNZ2600 produced mainly lactate under noninduction conditions (no nisin). However, when the heterologous NADH oxidase overproduction was induced with nisin, the metabolism of this strain progressively switched from homolactic to mixed-acid fermentation (Table 1). The magnitude of these shifts was directly dependent on the level of NADH oxidase overproduction. The most pronounced shift from homolactic towards mixed-acid fermentation coincided with the maximum NADH oxidase overproduction (1.2 U of nisin ml⁻¹) (Table 1). Products other than lactate amounted to 83% of the fermented glucose, with 28% of the pyruvate converted to acetate and 55% converted to acetoin or diacetyl (Table 1). The measured concentrations of fermentation products were highly reproducible in the duplicate samples, with maximum differences of only 5% in the values. At an initial growth pH of 6.0, induction with only 0.1 U of nisin ml⁻¹ (Table 2) rapidly induced a high level of NADH oxidase overproduction, and a pronounced shift towards mixed-acid fermentation was observed (Table 2). Despite the lower NADH oxidase activity reached at the highest level of induction relative to the situation at pH 7.0, pyruvate flux redistribution was almost identical at both initial pHs (Tables 1 and 2). Products other than lactate represented 85% of the fermented glucose, with 21% of the pyruvate converted to acetate and 64% converted to acetoin or diacetyl (Table 2). NADH oxidase overproduction decreased at nisin induction levels higher than 1.2 U ml⁻¹ at pH 7.0 and 0.4 U ml⁻¹ at pH 6.0. Consequently, homolactic fermentation was gradually restored (Tables 1 and 2). This was not a result of an effect on growth rates (0.69 at pH 7.0 and 0.79 at pH 6.0), which were identical under all nisin concentrations. LDH activities were measured and were very similar (8 U/mg) under all induction levels and conditions, indicating that the observed diminished flux through LDH was due to direct oxidation of NADH by NADH oxidase rather than an inactivation per se of LDH.

NADH/NAD ratios. The cultures described in Tables 1 and 2 could not be directly compared for NADH/NAD ratios be-

TABLE 2. Glucose fermentation pattern of *L. lactis* NZ9800(pNZ2600) at initial growth pH of 6.0

Amt of nisin added (U/ml)	Final pH	Amt of product produced (mM)			Amt of NADH oxidase overproduced (U/mg)*
		Lactate	Acetate	Acetoin or diacetyl	
0	5.03	22.5	4.8	ND ^b	0.21
0.1	5.64	7.8	5.3	7.5	8.48
0.2	5.78	5.2	5.4	8.2	10.41
0.4	5.78	4.3	5.9	8.8	14.35
0.8	5.75	5.0	5.7	8.5	14.23
1	5.69	5.9	5.4	7.6	12.74
1.2	5.46	10.8	3.9	5.3	10.49
1.4	5.22	16.6	3.4	2.8	4.64

* Overproduction of the heterologous H₂O-forming NADH oxidase at an initial growth pH of 6.0.

^b ND, not detected.

cause of the large differences in culture pH, resulting in large variation in NADH levels (23). To avoid this problem, cells were cultivated in separate experiments, at initial pH 7.0, aerobically and anaerobically, in the presence and absence of 1.2 U of nisin per ml. The cultures were harvested in early exponential phase to avoid excessive acidification of the culture medium. Increased activity of NADH oxidase lead to clear changes in the NADH/NAD ratios in the *L. lactis* cell. Cells with induced high NADH oxidase activity (30 U/mg) were compared aerobically and anaerobically. NADH/NAD ratios in the cells dropped dramatically by switching from anaerobic to aerobic conditions, from 0.74 to 0.46. The anaerobic and aerobic cultures were harvested at comparable pH values (5.9 and 6.1, respectively). In control experiments with cultures without induced high NADH oxidase activity, no changes in NADH/NAD ratios were observed when cultures were switched from anaerobic to aerobic conditions (data not shown).

Optimization of acetoin or diacetyl production from glucose by further activation of NADH oxidase. To demonstrate the applicability of changing metabolic flux via cofactor engineering, we tried to improve the production of diacetyl and acetoin via the α -ALS pathway. Under optimum NADH oxidase overproduction conditions, 57% (Table 1) to 60% (Table 2) of the fermented glucose was converted into acetoin or diacetyl via α -ALS. It seemed possible that the nisin-induced cells of *L. lactis* NZ9800 cells harboring pNZ2600 might lack endogenous FAD to fully satisfy the demand of this cofactor necessary for the activity of the overproduced NADH oxidase. In order to complement this lack, FAD was exogenously added to medium with an initial pH of 6.0, besides the nisin necessary for induction. The fermentation results without added FAD were very similar to the results presented in Table 2, although NADH oxidase activities were lower and total fermentation products were higher due to unavoidable variations in medium preparation and sterilization, resulting in slightly lower nisin concentrations, slightly higher initial glucose concentrations, and slightly higher cell densities (data not shown). In the control experiments, it was shown that the addition of FAD had no effect on product formation in noninduced cultures (Table 3). The addition of FAD to the induced cultures resulted in increased activity of the overproduced NADH oxidase, and consequently, a more efficient pyruvate flux redistribution was observed compared to that in the same medium lacking FAD. Under these conditions, lactate production was abolished, and the pyruvate was not channeled via α -ALS, instead, resulting in an increase in acetoin or diacetyl production from 57 to 74% of

TABLE 3. Effect of exogenously added FAD on the metabolism of *L. lactis* NZ9800(pNZ2600)^a

Product added ^b	Final pH	Amt of product produced (mM)			Amt of NADH oxidase overproduced (U/mg) ^c
		Lactate	Acetate	Acetoin or diacetyl	
Control with FAD	4.94	27.5	4.7	0.2	0.31
Control with nisin	5.58	9.0	5.9	9.9	8.29
FAD	5.65	ND ^d	6.8	11.7	13.67

^a All experiments were carried out at an initial pH of 6.0. Formate and butanediol were not detected.

^b Nisin (0.4 U ml⁻¹) was not added to the control with FAD. FAD (2 µg ml⁻¹) was not added to the control with nisin.

^c Overproduction of the heterologous H₂O-forming NADH oxidase.

^d ND, not detected.

the fermented glucose, with acetate production remaining at the same level.

DISCUSSION

The cloning of the *S. mutans* *nax-2* gene, coding for the H₂O-forming (nontoxic) NADH oxidase, under the control of the *nisA* promoter in *L. lactis*, provides a powerful tool with which to modulate metabolism in this microorganism. This engineered system allowed a fine tuning of NADH oxidase overexpression in such a way that deliberate and controlled variations of the NADH oxidase activity could be obtained by the addition of nisin. These variations correlated with a gradual shift from homolactic (noninduction) to mixed-acid fermentation (induction). The main effect of overproducing the NADH oxidase was an observed decrease in the NADH/NAD ratio under aerobic conditions. This decrease could lead to high acetate production by the pyruvate dehydrogenase (PDH) activity, since this enzyme complex has been reported to be very sensitive to a high NADH/NAD ratio (23, 24). However acetate production was not affected by the level of NADH oxidase overproduction (Tables 1 and 2), indicating that flux through PDH was limited. This limitation in the pyruvate metabolism correlates well with the previously reported poor PDH expression at high glycolytic flux (24). Nevertheless, we have shown that the magnitude of the shift towards mixed-acid fermentation was dependent on the level of NADH oxidase overexpression. This result, together with the observed flux limitation through PDH, demonstrated that the diminished flux through LDH, as seen by the decrease in lactate production, could only be provoked by the NADH oxidation catalyzed by the overproduced NADH oxidase. The pyruvate excess created under these conditions was accommodated by the α -ALS. This enzyme is known to efficiently convert pyruvate into α -acetolactate (and subsequently acetoin and diacetyl) even at an initial growth pH of 7.0, which is not optimal for its activity (24). At an initial pH of 6.0, the cytoplasmic pH of *L. lactis* is known to be significantly lower (13) and closer to the optimum for α -ALS activity, and therefore this enzyme could compete for pyruvate more efficiently than at neutral pH, as shown by the higher acetoin and diacetyl production observed at pH 6.0 (with lower NADH oxidase levels) compared to at pH 7.0 (Tables 1 and 2). These results showed that α -ALS behaved as a flexible branchpoint only under conditions leading to a pyruvate excess, as would be predicted by its low affinity for pyruvate ($K_m = 50$ mM) (24).

Recently, it was proposed that the low NADH/NAD ratio generated in vivo during diminished glycolytic flux played the

predominant role in the observed shift from homolactic to mixed-acid fermentation under anaerobic conditions (9). It was proposed that a strong inhibition of LDH was effected by this low ratio. However, under these conditions, pyruvate formate lyase (PFL) was operative because of the observed low concentrations of triose phosphate pools. It is feasible that PFL, like PDH, is highly expressed at a low glycolytic flux. Therefore, PFL could play a very important role in pyruvate flux redistribution under anaerobic conditions. Under aerobic conditions, PFL regulation, by the triose phosphate pools, will play no role, since this enzyme is well known to be completely inhibited by oxygen (14). This was evidenced by the absence of formate and ethanol production in the aerobic fermentations. Consequently, the metabolic effects of NADH oxidase overproduction described here cannot be ascribed to a possible influence of PFL on pyruvate flux redistribution.

Differences in the efficiency of the overexpression system were found to depend on the initial pH of the cultures. At an initial growth pH of 6.0, smaller amounts of nisin were required to optimize NADH oxidase overproduction, but maximum NADH oxidase activity was lower than that found at pH 7.0. These differences revealed a more efficient operation of the nisin-controlled overexpression system at low pH, but also an earlier saturation than at neutral pH. Independent of the initial growth pH, the system reached a plateau after which there was a progressive decrease in NADH oxidase overproduction. This observed shutoff mechanism could be a result of a physiological effect on the *L. lactis* cells by the higher nisin concentrations, although no decreases in growth rates were observed. Since nisin is more active at lower pH, this physiological effect leading to shutoff would occur at lower concentrations at pH 6.0 than at pH 7.0.

Addition of FAD to the medium increased the activity of the overproduced NADH oxidase. Consequently, lactate production was further decreased and even completely abolished. LDH activity was still found under these conditions, which further indicates that an efficient NADH oxidation was solely responsible for the abolished flux through LDH. The pyruvate, which accumulated because of the lack of NADH necessary for reduction to lactate, was mainly rerouted towards acetoin and diacetyl production and not to acetate. This is again a clear indication that flux through PDH was saturated at a high dilution rate.

This study demonstrated that metabolic engineering on the level of oxidation of the key cofactor NADH can change *L. lactis* from a homolactic bacterium to a highly acetoin- or diacetyl-producing bacterium. The observed rerouting of metabolism towards acetoin or diacetyl production by engineering on the level of cofactor regeneration is clearly more effective than the previously described metabolic engineering strategies focused on changes in activity of the pyruvate-converting enzymes (2, 10, 22, 25). Optimization of activity of the overproduced NADH oxidase by addition of FAD under aerobic growth conditions changed pyruvate metabolism in such a way that only two (PDH and α -ALS) of the four (LDH, PFL, PDH, and α -ALS) possible enzymes converting pyruvate were operative (Fig. 1). Metabolic engineering strategies directed to modulate key cofactors such as NADH could be a more effective way to manipulate metabolism than strategies involving the engineering of several branchpoints in the network.

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